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Fluorescent marked mosquito offer a method for tracking and study mosquito behaviour

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Abstract
Despite intensive research on vector-borne diseases, there remains a bias toward bionomics, ento-epidemiology, and operational research. In particular, relatively few studies have focused on vector behaviour and the use of labelling/marking techniques to do so. This manuscript dealt with a unique and easy mass marking technique, done using two different fluorescent colour dyes – Rhodamine and Fluorescein on *Culex quinquefasciatus*. We prepared 10% sugar solution in water containing these two dyes and fed the mosquitoes. Mosquito abdomen becomes marked with respective colour after feeding. Thus, by using this simple feeding technique, we could address and eliminate most of the challenges of insect marking observed in the past. Further, we found no significant difference in survival between mosquitoes marked with fluorescent dyes and the control group of mosquitoes. We also did not find any impact of two marking dyes on the reproductive health and life cycle of the mosquitoes. This method of colour marking can be used for studies related to the mark-release-recapture of mosquitoes, track free flying mosquito's behaviour in a dark room, simultaneous bio-toxicity or repellent experiment of different populations or strains of same species together in a single experiment and many more novel applications.

Keywords: Mosquito marking, insect tracking, mosquito behaviour, mark release recapture, fluorescent dye

1. Introduction
Vector-borne diseases, transferred from person-to-person by mosquitoes and other arthropods, resulting in illness and even death, prevents the holistic development of countries and economies in tropical and subtropical regions. Research is being conducted to find effective and economical options to prevent and control these diseases. Mainly bed nets, chemical insecticides in the household and public health domain and insect repellents are being used for preventing vector-borne diseases. However, to protect more people from bites of mosquitoes, research into an effective repellent as well as assessing mosquito behaviour (flight patterns, landing sites, bio-toxicity, ability to find holes in bed nets etc.) is of prime importance. Observation of mosquito in dark present a good opportunity to study mosquito behaviour and possibly support next-generation strategies of vector control. But, tracking wild mosquitoes poses challenges. Here, we are presenting a simple methodology by labelling/marking mosquito using fluorescent dyes.

Although the quest for marking insects is on for long, the search for an all-inclusive marker has remained a challenge. Therefore, the scientists experimented with diverse methods for marking of insects, which was reviewed previously [1]. Some of these methods include marking by (a) labelling, (b) mutilation, (c) with paint and ink, (d) with dust (otherwise called "powders" marking), (e) with colours, (f) genetic/hereditary (g) radioactive-isotope marking, (h) marking with rare- or trace-element [1].

An ideal marking material should be non-toxic (to the insect and the environment), distinctly identifiable, readily available, easy to apply and economical. The marker should not knock down the insect or chafe the insect, nor should it influence its ordinary physiology, behaviour, development, propagation, or life expectancy. For this reason, thorough studies on the impact of marker over insects should be conducted before a new marker can be used to study insect.

Fluorescent dyes offer an interesting avenue to mark insects, as it is non-toxic, readily available, provides a strong and attractive visual effect, available in different colours and cost-effective [1].

In the past, these dyes have been used in marking mosquitoes by physical dusting on the insects [2–5]. Mosquitoes that were successfully marked with dusted fluorescent dyes have shown to have no effect on the behaviour and survival [2]. But the process of a physical dusting of the dye may cause some behavioural discomfort to the insect due to physical damage and carries the risk of transferring the dye to an unmarked insect [1].

Here we address many of the challenges of insect marking observed in all current marker techniques by a simple technique using two fluorescent dyes – Rhodamine and Fluorescein. In our technique, we use the laboratory feeding habits of the mosquitoes to feed the dye to the mosquito. Using the different coloured dyes, even species that are closely related are also distinguished. The impact of ingestion of the dyes on the behaviour and physiology of mosquito (e.g., survival, reproductive health, the toxicity of the mosquitoes) have also been investigated. This methodology in our opinion will be very useful in understanding mosquito behaviour and research on disease transmission and control.

2. Materials and Methods

2.1 Mosquitoes and Fluorescent Chemicals

Two strains of *Culex quinquefasciatus* reared in the permanent laboratory colony of Godrej Consumer Products Ltd were used in this study. The temperature and humidity in the insectary are kept between 27 ± 2 °C and 70-80% respectively. Larvae were fed on ground soya bean powder mixed with vitamin B supplement and the adult mosquitoes were fed with 10% sugar solution soaked into laboratory filter paper. For regular colonization purpose, the adult mosquitoes are given blood meal after 6-8 days after adult emergence. A pool of freshly emerged adult mosquitoes (both male and female) were collected and taken for this study.

We used two water-soluble fluorescent dyes: Rhodamine and Fluorescein for the marking experiments. Rhodamine dye was obtained from Krishna Chemicals Ltd (Gujrat, India) and Fluorescein was obtained from Merck India Ltd.

2.2 Preparation of the fluorescent dye for feeding to mosquitoes

Rhodamin (0.1%) and Fluorescein (0.01%) solutions were prepared in water and sugar added to 100 ml of these solutions to prepare a final 10% sugar of respective dye. These sugar containing dye solution were used to feed mosquitoes through soaking in laboratory filter paper. The adult mosquitoes were allowed to feed on the Fluorescein and Rhodamin sugar solution as feed in separate cages. The dye-solution fed mosquitoes were captured in collection tubes and visualised in a black box with a UV Illuminator (365nm).

2.3 Experiment 1: Studying the feeding rates and affinity of the mosquitoes to different fluorescent feed

We studied if there is any specific affinity of the mosquitoes towards the two artificial dye mixed with sugar diets. *Feeding Rate study:* a total of 100 (1 to 2 day old) adult female mosquitoes from the regular mosquito rearing pool were collected and starved them for 24 hrs before start the study of feeding rate of adult mosquitoes towards a specific dye mixed sugar diet. A total of 50 mosquitoes each were placed into two separate cages. One cage was provided with Rhodamin mixed sugar diet and the other was provided with Fluorescein mixed sugar diet. The number of mosquitoes fed was recorded visually since the respective colour is easily visible in the abdomen of the mosquitoes. *Affinity study:* we studied the affinity of the mosquitoes towards the specific dye solution in the presence of standard 10% sugar diet. A total of 50 (1 to 2 day old) female mosquitoes were collected and starved for 24 hrs before introducing them in a single cage provided with three diets (two marker diets and one only sugar diet). The number of mosquitoes fed on each diet was recorded visually.

2.4 Experiment 2: Effect of marking on mosquito survival

The effect of fluorescent dyes on the survival of mosquitoes was investigated by counting the number of mosquitoes that found dead everyday morning. Adult mortality data was recorded until next cycle of blood feeding. We have taken 50 adult female and 100 male (both 24 hours starved) mosquitoes each in three cages and provided them with Fluorescein, Rhodamin mixed sugar diets and only 10% sugar solution (a control cage) respectively. Then, the dead mosquitoes were counted every day and recorded up to 7th day. (Day 4th data not available due to some unavoidable logistics issue that was beyond our control).

2.5 Experiment 3: Effect of marking on mosquito's response to blood feeding

We examined the effect of the fluorescent marking on mosquito's response to blood feeding. A total of 50 each gravid female mosquitoes were collected into three separate cages and reared on Rhodamin diet, Fluorescein diet and only 10% sugar solution diet (uncoloured control) for 48hrs. These marked and unmarked gravid female mosquitoes were provided with blood meal using Hemotek artificial blood feeding machine after 6 hours of starvation and counted the number of blood-fed mosquitoes in each cage.

2.6 Experiment 4: Effect of marking on reproductive health and F1 adult mortality of the dye fed mosquitoes

We checked the reproductive health of the marked mosquitoes by feeding adult mosquitoes (50 females and 100 male) with the Rhodamin diet, Fluorescein diet and unmarked sugar diet (as control) and allowed them to breed. The mosquitoes were given blood meal after 6 days of feeding to allow egg laying. Observations of the different life stages were recorded and compared to the control experiment to understand the reproductive health of the mosquitoes after colour marking using dye in the diet.

2.7 Experiment 5: Effect of marking on the contact toxicity of mosquitoes against insecticide molecule

We tested whether marking of mosquitoes using fluorescent dye mix diet influence the toxicity response of the mosquitoes towards insecticides. For this purpose, we have used Transfluthrin (purchased from Sigma-Aldrich) as insecticide molecule and bottle as an apparatus for exposing the mosquitoes in a different dosage. This is being adopted from *CDC Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay* [6] with few minor and reasonable modifications. The reagent bottles (Scott-Duran) of 500 ml with narrow mouth diameter is 86 mm and height of 181 mm were used to carry out the tests. All tests were done at 25% ± 2 °C and 60% ± 10% relative humidity. Mosquitoes reared on Rhodamin diet, Fluorescein diet and

only sugar diet were exposed to 2 µg/ml concentration of Transfluthrin (internally determined diagnostic dose – data not shown in this experiment) and recorded the knockdown data for one-hour exposure. Post-exposure, the mosquitoes were transferred to cages with respective food to record the mortality after 24 hours.

2.8 Statistical analysis

Differences in survival between mosquitoes coloured with the Rhodamine and Fluorescein compared to control sugar solution (uncoloured) was tested. The affinity of mosquitoes towards different fluorescent diets was analysed using ANOVA. Bio-toxicity data was analysed using log-dose probit analysis. We used StatsDirect Statistical Software v2.7.9 (07/09/2012) for all data analysis.

3. Result and Discussion

Adult mosquitoes need sugar for their survival and growth. Rhodamine and Fluorescein were individually mixed with 10% sugar solution and the adult mosquitoes were fed. Mosquito abdomen becomes marked with respective colour after feeding. The abdomen of Rhodamin fed mosquitoes become red colour and Fluorescein fed become green colour whereas control (fed with 10% sugar solution only) remain unmarked (Fig. 1).

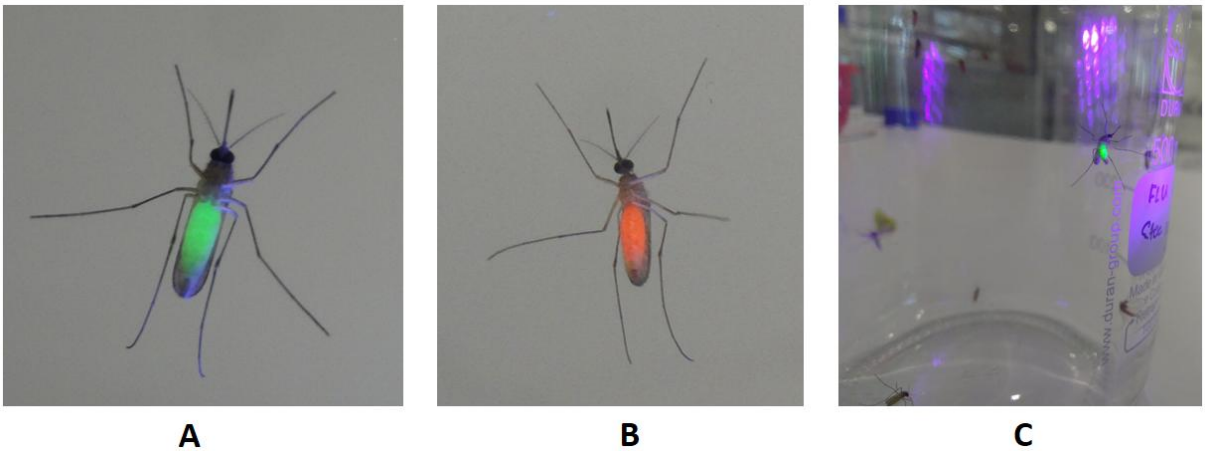

Fig 1: *Culex* mosquito marked/labelled with fluorescent dyes through adult diet. (A) Marked with Fluorescein producing green fluorescent colour (B) Marked with Rhodamin dye producing red colour after illuminating with blue-black light. (C) Marked mosquitoes tested in bottle assay.

Experiment – 1: We tested that observed difference in feeding rates to Rhodamine and Fluorescein was not statistically significant (P = 0.1088, F = 7.72) (Fig. 2). The possibility of any difference in the affinity of mosquitoes towards Rhodamin or Fluorescein was tested and observed that mosquito's affinity towards these two dyes (mixed with sugar diet) is significantly different (P = 0.0082, F = 7.36). The mosquito has a preference towards Rhodamin mixed sugar diet over Fluorescein (Fig. 3), which is consistent with our observation of feeding rate study (Fig. 2).

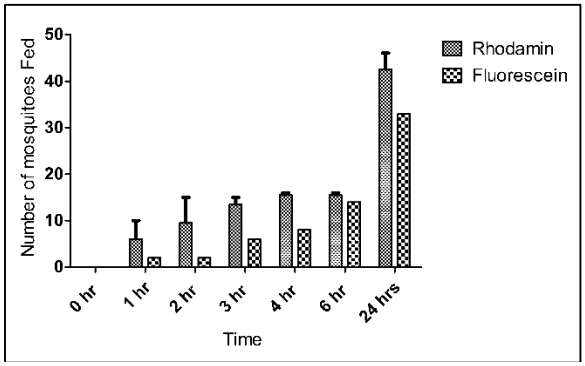

Fig 2: Mosquito feeding rates over time on Rhodamine and Fluorescein dye mixed sugar solution

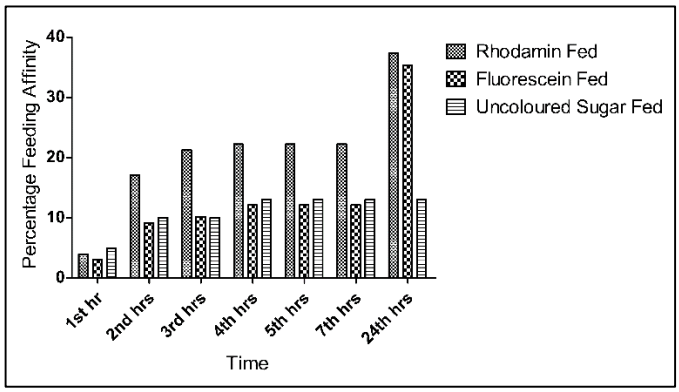

Fig 3: Mosquito affinity towards marking diets. The mosquito has a preference towards Rhodamin mixed sugar diet over Fluorescein.

Experiment – 2: We tested daily mortality of mosquitoes that were fed with Rhodamin, Fluorescein and only sugar diet (Fig. 4). No significant difference in survival between mosquitoes reared on two fluorescent dyes and the control group of mosquitoes (P = 0.121, F = 2.01) was observed. This finding is in line with another study where mosquitoes were marked physically with fluorescent dye found no significant difference in survival among control and colour treatment [2].

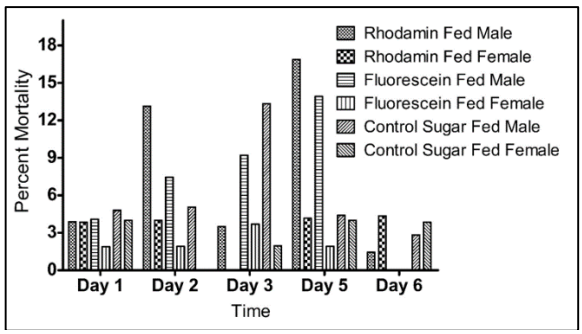

Fig. 4. Mosquito survival after feeding to fluorescent dye mixed diets.

Experiment – 3 and 4: We tested the effect of the fluorescent marking on mosquito's response to blood feeding and the reproductive health. Table 1 represents the data of blood feeding affinity and reproductive health of mosquitoes marked with two fluorescent dyes as well as unmarked control. We found no significant impact of these two marking dyes on the reproductive health and life cycle of the mosquitoes. The average blood feeding in three diets ranges from 53.33% to 65.63% and mosquitoes laid 6-8 eggs post blood feeding. This confirmed that feeding mosquitoes with fluorescent dye did not affect their normal reproductive physiology.

Table 1: Results of Experiment – 3 and 4 are shown in below table. Different parameters are checked to study the effect of marking on mosquito's response to blood feeding and their reproductive health.

Parameters	Rhodamin mixed sugar	Fluorescein mixed sugar	Only sugar (Un-colour control)
Average blood feeding affinity (%)	65.63	53.33	53.33
Number of egg laid	8	8	6
Average time for egg laying after blood meal	2 days	2 days	2 days
Average time for eggs to hatch	1 day	1 day	1 day
Average time for pupation after egg hatch	9 days	9 days	10 days
Average time for Adult emergence after pupation	2 days	2 days	1 day

Experiment – 5: We tested whether marking of mosquitoes has any impact on the insecticide toxicity. Transfluthrin was used for this study and no significant difference in bio-toxicity between two fluorescent dyes and control (P = 0.1053, F = 3.35) was observed. Table 2 represents the results obtained from the bio-toxicity assay, where it shows that time to knock down 50% of test mosquitoes (KT_{50}) needs 55.5, 57.96 and 47.74 minutes (mortality = 36%, 32% and 34%) in Rhodamin, Fluorescein and unmarked (control) mosquitoes respectively.

Table 2: Bio-toxicity Assay of marked and unmarked mosquitoes against Transfluthrin.

Type of Mosquitoes	KT_{50}	95% CI	KT_{95}	95% CI	Chi^2	t for slope	Degree of Freedom	% mortality post 24 hrs
Rhodamin mixed sugar fed	55.5	48.68 – 68.24	131.43	96.23 – 245.68	0.823 (P = 0.991)	5.624 (P = 0.0014)	6	36
Fluorescein mixed fed	57.96	48.45 – 77.37	196.39	126.92 – 459.16	3.711 (P = 0.72)	5.904 (P = 0.001)	6	32
Only Sugar mixed fed (Control)	47.74	41.71 – 57.3	132.88	97.21 – 231.88	2.822 (P = 0.83)	6.62 (P = 0.0006)	6	34

In the past, many Lepidoptera pests were marked with various dyes by adding the dye directly into larval diets [7, 8]. Rhodamine B was successfully added to larval diets for marking adult moths too [9, 10]. There are certain limitations in the various methods that are used for marking individual insects, as labelling individual insect is repetitive, tedious and could overwhelm the insects. Some marking techniques (e.g., mutilation, painting, dust marking etc.) require broad treatment of individual insects with considerable size, which can make the methodology time-consuming, hurtful to the insect and unrealistic for mass marking of insects [11–14]. On the other hand, genetic marking is uncommon and needs some consistent generations in the laboratory to develop with the assurance of having no harmful consequences for insect's physiology or behaviour. Radioactive isotope marking is not feasible due to stricter ecological security laws. Trace elemental marking has the limitation of detection, which is expensive and time-consuming.

4. Conclusion

Unlike the above methods which are less convenient as well as being laden with many limitations, our results show that the technique used here for marking mosquitoes did not alter the mosquitos' normal physiology and behaviour. Additionally, the method described in this paper is nontoxic (to the insect as well as environment), effortless, easily identifiable, strong and economical. This method of colour marking could be used for tracking mosquito behaviour in dark, studies related to the mark-release capture of mosquitoes; study visual differentiation between closely related species of mosquitoes with the help of marking techniques, studying closely related but different species or different strains of same species together in one experiment etc. This method could simplify the process of studying closely related species under identical environment and further our knowledge on mosquito behaviour and control.

5. Author Contribution

Dipon developed methodology, curated data, analysed results. Siva being the project administration and resources provider. Siva also reviewed and edited the original draft. Manas conceptualized the study, developed methodology, supervised experiments, analysed results and drafted manuscript.

6. Acknowledgement
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